This is the **Exhibit B** referred to in the Declaration under 37 C.F.R. Section 1.132 of ANTONY WILKS BURGESS dated June 14, 2006.

[29] Generation of Antibodies and Assays for Transforming Growth Factor β

By CATHERINE LUCAS, BRIAN M. FENDLY, VENKAT R. MUKKU, WAI LEE WONG, and MICHAEL A. PALLADINO

Introduction

Transforming growth factor β (TGF-β) is a growth factor which, like transforming growth factor α (TGF-α), was discovered in an assay evaluating cellular transformation of immortalized nonneoplastic fibroblasts.[1,2] TGF-β, however, is structurally unrelated to TGF-α and binds to distinct receptors. It is a disulfide-linked dimer of two identical 112 amino acid polypeptides, each derived from a larger secreted polypeptide. Protein biochemical analysis and cDNA cloning have established the existence of at least three human TGF-β species, which are structurally closely related to each other.[3-6] TGF-β stimulates or inhibits the proliferation of cells, depending on the cell type and the physiological conditions. A major activity of TGF-β resides in its ability to induce extracellular matrix formation. These various biological activities strongly suggest that TGF-β plays a crucial role in cell proliferation and differentiation *in vivo*. The generation of antibodies to TGF-β was of critical importance in studying the functionality of TGF-β in normal physiology and malignant transformation, as well as in developing precise and specific assays. The development of such antibodies and assays are described below.

The assays which initially led to the discovery of TGF-β are based on the ability of TGF-β to induce anchorage independence in some immortalized fibroblasts, either in the presence or the absence of epidermal growth

[1] A. B. Roberts and M. B. Sporn, *in* "Peptide Growth Factors and Their Receptors" (M. B. Sporn and A. B. Roberts, eds.), Handbook of Experimental Pharmacology, Vol. 95/1, p. 3. Springer-Verlag, Heidelberg, 1990.

[2] M. B. Sporn, A. B. Roberts, L. M. Wakefield, and B. de Crombrugghe, *J. Cell Biol.* **105**, 1039 (1987).

[3] R. Derynck, P. B. Lindquist, A. Lee, D. Wen, J. Tamm, J. L. Graycar, L. Rhee, A. J. Mason, D. A. Miller, R. J. Coffey, H. L. Moses, and E. Y. Chen, *EMBO J.* **7**, 3737 (1988).

[4] S. B. Jakewlew, P. J. Dillard, P. Kondaiah, M. B. Sporn, and A. B. Roberts, *Mol. Endocrinol.* **2**, 747 (1988).

[5] P. van Dyke, T. P. Hansen, K. K. Iwata, C. Pieler, and J. G. Foulkes, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4715 (1988).

[6] R. de Martin, B. Haendler, R. Hofer-Warbinek, H. Gaugitsch, M. Wrann, H. Schlusener, J. M. Seifert, S. Bodmer, A. Fontana, and E. Hofer, *EMBO J.* **6**, 3673 (1987).

factor (EGF). These assays, however, are not specific for this growth factor and are subject to interference or synergism by other factors. Thus, they do not allow reliable quantitation of TGF-β in crude extracts or conditioned media. In addition, as discussed above, there are multiple TGF-β species which are structurally closely related and have similar activities,[3-6] and TGF-β is biosynthesized as a protein-bound, latent, inactive form.[7-10] An ideal assay for TGF-β should be able to quantitate the factor in impure preparations and discriminate between the different TGF-β species as well as between the active and latent complexed forms. Several laboratories have recently developed receptor-binding assays based on a competition of the TGF-β in the sample with ^{125}I-labeled TGF-β for binding to the TGF-β receptors.[11-13] These assays allow reliable quantitation of active TGF-β but do not discriminate between the different TGF-β species. The total amount of TGF-β in test samples can be quantitated after activation of TGF-β with acidic pH, followed by neutralization. We describe the production of monoclonal antibodies specific for TGF-β1 and the radiolabeling of this protein. These reagents together with human TGF-β1 from recombinant sources[14] were used for the development of a double-antibody enzyme immunoassay and a radioimmunoassay specific for TGF-β1. We also describe a radioreceptor assay and a bioactivity assay for TGF-β.

Radioiodination of Transforming Growth Factor β1

Examination of the literature indicates that most laboratories prepare the ^{125}I-labeled TGF-β needed for assays using the chloramine-T method.[15,16] We have compared several methods for iodination of TGF-β

[7] D. A. Lawrence, R. Pircher, and P. Jullien, *Biochem. Biophys. Res. Commun.* **133,** 1026 (1985).
[8] I. A. Silver, R. J. Murrills, and D. J. Etherington, *Exp. Cell Res.* **175,** 266 (1988).
[9] R. O. C. Oreffo, G. R. Mundy, S. M. Seyedin, and L. F. Bonewald, *Biochem. Biophys. Res. Commun.* **158,** 817 (1989).
[10] Y. Pilatte, J. Bignon, and C. R. Lambre, *Biochim. Biophys. Acta* **923,** 150 (1987).
[11] B. O. Fanger and M. B. Sporn, *Anal. Biochem.* **156,** 444 (1986).
[12] P. R. Segarini, A. B. Roberts, D. M. Rosen, and S. M. Seyedin, *J. Biol. Chem.* **262,** 14655 (1987).
[13] L. M. Wakefield, L. M. Smith, T. Masui, C. C. Harris, and M. B. Sporn, *J. Cell Biol.* **105,** 965 (1987).
[14] R. Derynck, J. A. Jarrett, E. Y. Chen, D. H. Eaton, J. R. Bell, R. K. Assoian, A. B. Roberts, M. B. Sporn, and D. V. Goeddel, *Nature (London)* **316,** 701 (1985).
[15] W. M. Hunter and F. C. Greenwood, *Nature (London)* **194,** 495 (1962).
[16] C. A. Frolik, L. M. Wakefield, D. M. Smith, and M. B. Sporn, *J. Biol. Chem.* **259,** 10995 (1984).

and found that a high specific radioactivity was obtained with both the chloramine-T[15] and lactoperoxidase[17] methods. Using these methods the ^{125}I-labeled TGF-β remained biologically active. The lactoperoxidase iodinations generally resulted in a higher specific radioactivity than the chloramine-T method, but the latter method generally resulted in a better purity as assessed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Both methods of iodination are described below. Separation of iodinated TGF-β from free sodium iodide was best performed by HPLC, although separation by Sephadex G-100 filtration was adequate. The TGF-β used in these preparations was recombinant human TGF-β1, derived from mammalian cells transfected with an expression vector.

Chloramine-T Iodination Procedure

Chloramine-T is a strong oxidant which is able to covalently link iodine to tyrosine residues of proteins. The iodination procedure is performed on ice at neutral pH and by three sequential additions of chloramine-T. Approximately 10 μl (1 mCi) of Na^{125}I (Amersham, Arlington Heights, IL; 13–17 mCi/μg) is added to 10 μg of TGF-β in 50 μl of iodination buffer (1.5 *M* potassium phosphate, pH 7.4). TGF-β is iodinated by 3 sequential additions of 20 μl of the chloramine-T solution [0.1 mg/ml chloramine-T (Kodak, Rochester, NY) in 50 m*M* sodium phosphate, pH 7.4]. Between each addition, the reaction mixture is incubated for 1.5–2 min, with occasional mixing. The reaction is stopped by adding 20 μl of 50 m*M* *N*-acetyl-L-tyrosine for 1 min, then 20 μl of 1 *M* KI for 1 min, and finally 200 μl of 8 *M* urea, pH 3.2. The tracer is purified by HPLC or by gel-filtration chromatography. The HPLC purification generally resulted in a better recovery (>90%) and a higher purity.

Lactoperoxidase Iodination

Lactoperoxidase is an oxidative enzyme which catalyzes the enzymatic iodination of tyrosine-containing proteins. This reaction is a mild procedure which can be carried out at room temperature over a wide pH range (4.0 to 8.5), an advantage for proteins with unusually high or low isoelectric points and limited solubility at neutral pH. We found purified TGF-β1 to be poorly soluble in aqueous solutions above pH 5.0 and therefore chose to perform the lactoperoxidase iodination at pH 4.5.

Ten micrograms of purified TGF-β1 (~0.5 nmol) in 35 μl of 20 m*M* acetic acid, pH 4.0, is placed in a microcentrifuge tube, and 5 μl of 0.5 *M* sodium phosphate buffer, pH 7.4, is added to adjust the pH of the solution.

[17] J. I. Thorell and B. G. Johansson, *Biochim. Biophys. Acta* **251**, 363 (1971).

Approximately 10 μl (1 mCi) of $Na^{125}I$ (Amersham; 13–17 mCi/μg) is added to the protein, then 5 μl (7 μg) of a freshly made solution of 1.4 mg lactoperoxidase (Sigma, St. Louis, MO) per milliliter phosphate-buffered saline (PBS), followed by 5 μl of 20 μ*M* H_2O_2. The tube is capped, gently agitated, and incubated at room temperature for 10 min with occasional gentle mixing. The reaction is terminated by the addition of 20 μl of a fresh solution of 0.5 *M* *N*-acetyl-L-tyrosine (Sigma), followed 2 min later by 20 μl of 0.5 *M* KI and 200 μl of 8 *M* urea to retrieve the tracer from the reaction tube. The ^{125}I-labeled TGF-β is then purified by HPLC or by size-exclusion chromatography as described below.

Chromatographic Purification of ^{125}I-Labeled Transforming Growth Factor β

Purification of the radiolabeled TGF-β took place on an LKB (Uppsala, Sweden) HPLC system (LKB 2150 pump and 2152 LC controller) using a reversed-phase HPLC column C_{18}-300 (Vydac, Alltech Associates, Deerfield, IL). After equilibrating the column with solution A (0.1% trifluoroacetic acid in water), 0.5 ml of the iodination mixture is injected onto the column, and 60 fractions of 0.5 ml each are collected into borosilicate tubes, each containing 50 μl of 10% bovine serum albumin (BSA). A stepwise gradient is then started by adding solution B (0.08% trifluoroacetic acid in acetonitrile) to 25% over 5 min, then to 50% over the following 25 min, then to 100% over the following 5 min. The column is equilibrated and subsequently stored in 100% solution B. Five-microliter fractions are taken from each tube and counted in a γ counter to identify the iodinated protein peak (which elutes at 35–40% acetonitrile). Peak fractions are pooled, diluted to 5 ml with 1% BSA in 4 m*M* HCl, and stored in aliquots at −60° for approximately 1 month.

Sephadex G-100 Chromatography

A 1 × 30 cm glass Econo-column (Bio-Rad, Richmond, CA) is packed with Sephadex G-100, hydrated and degassed according to the manufacturer's recommendations. The column is equilibrated with approximately 50 ml running buffer (0.1% gelatin in 4 m*M* HCl). The iodination reaction is then loaded; the gel filtration takes place in running buffer, collecting 60 0.6-ml fractions. Five microliters is taken from each tube and counted in the γ counter to identify the iodinated protein peak in the void volume. The peak fractions are pooled and diluted with running buffer to a final volume of 5 ml. The iodinated TGF-β is stored in aliquots at −60° for approximately 1 month.

Trichloroacetic Acid Precipitation

The trichloroacetic acid (TCA) precipitability of radioactive counts gives a measure of the proportion of radiolabeled TGF-β relative to the unincorporated ^{125}I label remaining in the tracer preparation. A small amount of the ^{125}I-labeled TGF-β is diluted in tracer diluent (PBS, pH 7.4, containing 0.1% gelatin and 0.01% thimerosal or 0.5% BSA and 0.01% thimerosal) to obtain a minimum of 0.5 ml at 10^5–10^6 cpm/ml. The radioactivity in counts per minute (cpm)/100 μl is determined using an automatic γ counter. Two hundred microliters of the diluted radiolabeled preparation is added to 50 μl of 50% TCA in a microcentrifuge tube, mixed, and incubated for 30 min on ice. The precipitated sample is then centrifuged for 5 min at 13,000 g in a microcentrifuge at room temperature, and the amount of soluble counts in 100 μl of the supernatant is determined. The calculated percentage of TCA precipitability of the diluted radiolabeled TGF-β preparation should be more than 90%.

Using both iodination procedures described above, we have obtained ^{125}I-labeled TGF-β tracer preparations which typically had specific activities of 25 to 40 μCi/μg and were more than 90% TCA precipitable. The radiolabeled TGF-β preparations usually retained more than 90% of the bioactivity of the starting material, when tested in the bioassay described below. We found it to be very important in iodinating TGF-β to keep the protein at acidic pH, since TGF-β is not very soluble above pH 5.0. When the iodinations were initially less successful, we observed that the apparent loss of bioactivity was not so much due to iodination damage but rather attributable to loss of solubility of the protein. For this reason, we discontinued an initial evaluation of the Bolton–Hunter method of iodination and do not recommend it for TGF-β, because this method is best performed at basic pH[18] and results in considerable aggregation of the purified protein and very poor incorporation of the label.

Radioimmunoassay for Transforming Growth Factor β1

The radioimmunoassay (RIA) for TGF-β measures the displacement of the binding of radiolabeled TGF-β to a specific anti-TGF-β antibody by increasing concentrations of unlabeled TGF-β. Establishment of a standard curve using purified TGF-β1 shows that the signal in counts per minute in this competitive assay is inversely proportional to the concentration of unlabeled TGF-β1 present in the sample. The useful range of this assay is 6.25–200 ng/ml, with a sensitivity of 2.4 ng/ml.

[18] A. E. Bolton and W. M. Hunter, *Biochem. J.* **133**, 529 (1973).

One hundred microliters of each standard dilution (6.25–200 ng/ml TGF-β1), control, or test sample is added in duplicate into conical test tubes [four additional tubes contain only 100 μl of assay diluent (PBS containing 50 mg/liter BSA, 0.5 ml/liter Tween 20, 1 *M* NaCl, and 0.2 g/liter sodium azide) for nonspecific binding and zero reference]. One hundred microliters of ^{125}I-labeled TGF-β tracer is added to each tube, including two additional tubes for total counts. These two tubes are set aside until the end of the procedure. Then 100 μl of goat anti-TGF-β antibody appropriately diluted in assay diluent is added to all the tubes, except the two nonspecific binding tubes (which receive instead 100 μl of 2% normal goat serum). All tubes are mixed and incubated overnight at 4°. Immune complexes are precipitated with the addition of 1 ml of precipitation mixture [20 ml/liter donkey anti-goat IgG antibody (Pel-Freez, Rogers, AR) in PBS, pH 7.4, containing 40 g/liter polyethylene glycol (PEG) 8000 (Sigma) and 2 g/liter sodium azide] added to each tube for 1 hr at ambient temperature. The tubes are centrifuged for 20 min at 2000 *g* at 4° in a Beckman J-6B centrifuge, the supernatants are decanted, and the pellets are counted in a γ counter for 2 min each. The data are reduced with a four-parameter curve-fitting program based on an algorithm for least-squares estimation of nonlinear parameters.[19]

To determine the appropriate dilution of anti-TGF-β antibody for use in the assay, the antibody is first titrated as a series of 2-fold dilutions against the ^{125}I-labeled TGF-β tracer. The immune complexes are then precipitated as described in the procedure for the RIA. The three dilutions which precipitate 35 (±5)% of the counts are used to generate standard curves. The dilution which gives the most sensitivity is selected for the assay.

Radioreceptor Assay for Transforming Growth Factor β

The radioreceptor assay measures the ability of serial dilutions of TGF-β-containing samples to compete with ^{125}I-labeled TGF-β for the binding to the specific cell surface receptors. This assay is specific for biologically active TGF-β and does not detect the latent, protein-associated form of TGF-β,[7-10] unless previously acid-activated. The radioreceptor assay measures total binding to the different types of cell surface receptors and does not discriminate between the different TGF-β species. It is likely, however, that there are differences between the binding of the different TGF-β species to the three types of receptors, suggesting that some caution may be needed for the absolute quantitation of TGF-β. Our radioreceptor assay is done essentially as described by Wakefield *et al.*[13]

[19] D. W. Marquardt, *J. Soc. Ind. Appl. Math.* **11**, 431 (1963).

using A549 human lung carcinoma cells grown in monolayers. The range of the assay is 25–0.19 ng/ml.

A549 cells are grown at 37° with 5% CO_2 in high glucose Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) supplemented with 5% calf serum, 2 m*M* L-glutamine, and 10 m*M* sodium pyruvate. The cells are seeded at 2×10^5 cells/ml/well into the wells of 24-well cluster plates (Nunc, Naperville, IL) and grown in assay medium (i.e., Ham's F12 mixed 1 : 1 with low glucose DMEM and without $NaHCO_3$) for 24 hr until 90–95% confluency is reached. Four wells containing growth medium only are incubated under the same conditions and are the blanks in the assay. Monolayers are washed twice with 1 ml assay diluent (assay medium containing 1 g/liter BSA, 25 m*M* HEPES, pH 7.4, and 42.5 m*M* NaCl) immediately prior to sample addition. The TGF-β in the samples (100–200 μl each) is acid-activated by adding 10 μl of 1.2 *N* HCl for 30 min at ambient temperature and subsequently neutralized with 10 μl of a solution containing 1 *M* HEPES, pH 7.4, and 1.44 *M* NaOH. The activated samples are then diluted with assay diluent (final dilution 1 : 2.5 or higher). The standards (25–0.19 ng/ml in assay diluent), blanks (assay diluent), and activated test samples (0.5 ml each) are mixed in dilution tubes with 0.5 ml of ^{125}I-labeled TGF-β tracer diluted to 20,000 cpm/100 μl in assay diluent. In addition, four counting tubes containing 100 μl of diluted tracer alone are set aside for the determination of the total counts (from which percent binding will be determined for standards and samples).

The assay diluent is aspirated from the cell plates, and 200 μl of the activated test samples or control samples from the dilution tubes are added in quadruplicate to appropriate wells of the cell plates. The plates are covered and incubated for 2 hr at ambient temperature, rocking gently on a rocking platform, after which all wells are washed 4 times with 1 ml of tracer wash buffer (1 g/liter BSA in PBS, pH 7.4). Solubilization buffer (10% glycerol, 1% Triton X-100 in 25 m*M* HEPES, pH 7.4) is then added to each well (0.75 ml/well). Following incubation at 37° for 30 min on a plate rotator, the contents of each well are transferred to counting tubes and counted for 5 min in a γ counter. The concentration of TGF-β in the test samples is determined by comparison with the standard curve data in a four-parameter logistic curve-fitting program.

Production of Polyclonal Antibodies to Transforming Growth Factor β

The production of antibodies to TGF-β has been difficult, presumably because of its immunosuppressive activities and its very conserved polypeptide sequence, but successful attempts have been reported.[20] In order

[20] D. Danielpour, L. L. Dart, K. C. Flanders, A. B. Roberts, and M. B. Sporn, *J. Cell. Physiol.* **138**, 79 (1989).

to obtain polyclonal antibodies to TGF-β1, we have immunized a variety of animals (rabbits, goats, turkeys, and guinea pigs) by several immunization procedures, including alum-precipitated TGF-β1 or TGF-β1 in detox adjuvant (Ribi, Hamilton, MT) or Freund's adjuvant. Most animals developed titers to TGF-β1, but the titers were not very high nor sustained for long. Most striking was the finding that antibodies generated were directed to similar or competing epitopes. We were never able to develop a double-antibody ELISA with any combination of these antibodies. Since antibodies generally do not need to be purified for use in radioimmunoassays, we used unfractionated antiserum to TGF-β1 for this assay.

However, we also examined the affinity purification of rabbit anti-TGF-β1 antibodies for potential use in enzyme immunoassays. Before affinity chromatography, we isolated the γ-globulin fraction by two precipitations with 40% ammonium sulfate. In order to prepare an affinity column with TGF-β1, coupling conditions must be chosen which will be feasible at low pH, since TGF-β has only limited solubility at neutral or basic pH. In contrast, most coupling reactions of proteins to resins are best performed at alkaline pH (pH 8.0 to 9.0). However, we were successful with two procedures described below, with no obvious advantage of one over the other.

In the first procedure, we coupled TGF-β1 to aldehyde-activated polyethylene glycol-coated silica (Chromatochem, Merck, Darmstadt, Germany), which can be used in 100 m*M* sodium citrate, pH 3.5. Two milligrams of TGF-β1 in sodium citrate buffer is added to 0.62 g of resin rehydrated for 15 min with sodium citrate buffer and degassed. Ten milligrams of sodium cyanoborohydride ($NaCNBH_3$) in 0.2 ml citrate buffer is then immediately added to the slurry. Following an incubation of 30 min at 4°, the resin is centrifuged at room temperature for 5 min at 2000 *g* in a Micro-Centrifuge (Fisher Scientific, Fairlawn, NJ), washed 3 times with 5 ml each of citrate buffer, and blocked for 1 hr at 4° with 1 g glucosamine (Sigma) in 5 ml citrate buffer containing 10 mg $NaCNBH_3$.

Alternatively, TGF-β can be coupled to AH- or CH-Sepharose 4B (Pharmacia, Piscataway, NJ) or to Affi-Gel 102 aminoalkyl-agarose (Bio-Rad) via carbodiimide coupling. As coupling reagent we use diethylaminopropylcarbodiimide hydrochloride (EDAC, Bio-Rad) at 40 mg/ml in water, acidified to pH 4.5 with HCl. The coupling reagent (10 mg/ml resin) is added to 1 g of the resin (~4 ml) and 2 mg TGF-β1 in acidified water for an overnight incubation at 4°. After centrifugation for 5 min at 2000 *g* (Micro-Centrifuge, Micromedics Systems) and 3 washes with acidified water, the reactive resin is blocked for 1 hr at ambient temperature with 5 ml of 0.1 *M* sodium acetate, 0.5 *M* NaCl at pH 4.0.

Production of Monoclonal Antibodies to Transforming Growth Factor β1

As in the case of the polyclonal antisera, the production of monoclonal antibodies (MAb) to TGF-β1 was difficult, possibly owing to the immunosuppressive properties and the highly conserved amino acid sequence of this factor. We were not successful using intraperitoneal or subcutaneous immunization and conventional fusion protocols, whereas Dasch *et al.*[21] have succeeded using these methods. In our successful experiments, we immunized BALB/c mice with TGF-β1 using footpad injections, and their draining inguinal and popliteal lymph nodes were fused. This protocol resulted in a large percentage of positive clones with a specific fusion efficiency of 88%, whereas conventional fusion of splenocytes from mice immunized intraperitoneally or subcutaneously yielded low specific fusion efficiency, with predominantly antibodies of the IgM isotype.

Ten BALB/c mice are injected with 5 μg/dose of purified TGF-β1 in 100 μl detox adjuvant (Ribi) in the hind footpads on days 0, 3, 7, 10, and 14. On day 17 the animals are sacrificed, their draining inguinal and popliteal nodes removed, and the lymphocytes dissociated from the nodal stroma using stainless steel 200 mesh (Tylinter, Inc., Mentor, OH). The lymphocyte suspensions from all 10 mice are pooled and fused with the mouse myeloma line X63-Ag8.653,[22] using 50% PEG 4000 according to an established procedure.[23] The fused cells are inoculated in the wells of 96-well tissue culture plates (Falcon, Oxnard, CA) at a density of 2×10^5 cells/well followed by HAT (hypoxanthine, aminopterin, and thymidine) selection[24] on day 1 after fusion.

Parental hybridoma cultures are screened for production of antibody to TGF-β1 by indirect ELISA, using 96-well polystyrene microtiter plates (Immulon, Nunc) coated overnight at 4° with 100 μl/well of 1 μg/ml TGF-β1 in coating buffer (50 m*M* carbonate/bicarbonate, pH 9.6). Coated plates are washed 3 times with wash buffer (PBS, 0.05% Tween 20) and blocked for 1 hr at ambient temperature with ELISA diluent (PBS, pH 7.4, 0.5% BSA, 0.05% Tween 20, and 0.01% thimerosal). After 3 additional washes with wash buffer, hybridoma supernatants (100 μl) are added for 1 hr at ambient temperature. The plates are washed again 3 times with wash buffer, and bound antibodies are detected with 100 μl/well of goat anti-mouse IgG conjugated with horseradish peroxidase (Tago Inc., Bur-

[21] J. R. Dasch, D. R. Pace, W. Waegell, D. Inenaga, and L. Ellingsworth, *J. Immunol.* **142**, 1536 (1989).
[22] J. F. Kearney, A. Radbruch, B. Liesegang, and K. Rajewski, *J. Immunol.* **123**, 1548 (1979).
[23] V. Oi and L. Herzenberg, *in* "Selected Methods in Cellular Immunology" (B. Mishel and S. Shiigi, eds.), p. 351. Freeman, San Francisco, California, 1980.
[24] J. W. Littlefield, *Science* **145**, 709 (1964).

lingame, CA), freshly diluted 1 : 5000 in ELISA diluent. The plates are washed with wash buffer and developed with 100 μl/well of 2.2 m*M* *o*-phenylenediamine substrate in substrate buffer (50 m*M* sodium phosphate, 0.1 *M* sodium citrate, pH 5.0) containing 0.01% (v/v) H_2O_2. The reaction is stopped after 15 min with 100 μl/well of 4.5 *M* sulfuric acid, and plates are read at 492 nm with an automatic plate reader (Molecular Devices, Menlo Park, CA). Hybridoma cultures positive for antibody to TGF-β1 are cloned twice by limiting dilution and grown as ascites in pristane-primed BALB/c mice.[25] The MAbs are purified from ascites fluid by protein A-Sepharose affinity chromatography and eluted in 0.1 *M* acetic acid, 0.5 *M* NaCl, pH 2.4, according to established procedures.[26]

Three hybridomas were cloned and their monoclonal antibodies characterized in some detail. Each of the MAbs recognized the dimer form of TGF-β1 in immunoblots. MAb 2G7, an antibody of the $IgG_{1,\kappa}$ isotype, immunoprecipitated TGF-β1, -β2, and -β3 and neutralized all three species in a [^{3}H]thymidine uptake inhibition assay using a mink lung fibroblast cell line (described below). It had an affinity of 1.2×10^8 liters/mol for TGF-β1 as determined by Scatchard analysis using ^{125}I-labeled TGF-β1. MAb 4A11, also an $IgG_{1,\kappa}$ antibody, neutralized and immunoprecipitated only TGF-β1 and had a similar affinity constant. Finally MAb 12H5, an $IgG_{2b,\kappa}$ isotype, immunoprecipitated but was not neutralizing for TGF-β1, and it had an affinity constant of 5×10^7 liters/mol for this molecule.[27]

Enzyme-Linked Immunosorbent Assay for Quantitation of Transforming Growth Factor β1

Double-antibody enzyme-linked immunosorbent assays have been extensively described. The immunoassay we have developed for the quantitation of TGF-β1 is based on the classic procedure of Engvall and Perlmann[28] and uses two MAbs specific for TGF-β1, 12H5 and 4A11, for capture and detection, respectively. MAb 4A11, which neutralizes the activity of TGF-β1 *in vitro* (see bioassay procedure below), was conjugated to horseradish peroxidase according to established procedures.[29] The assay has a useful range of 40 to 0.63 ng/ml and a sensitivity of 0.63 ng/ml

[25] M. Potter, J. G. Humphrey, and J. L. Walter, *J. Natl. Cancer Inst.* **49**, 305 (1972).
[26] J. W. Goding, *J. Immunol. Methods* **20**, 241 (1978).
[27] C. Lucas, L. N. Bald, B. M. Fendly, M. Mora-Worms, I. S. Figari, E. J. Patzer, and M. A. Palladino, *J. Immunol.* **145**, 1415 (1990).
[28] E. Engvall and P. Perlmann, *Immunochemistry* **8**, 871 (1971).
[29] P. K. Nakane and G. B. Pierce, *J. Cell Biol.* **33**, 307 (1967).

as determined by the unpaired, one-tailed *t*-test method of Rodbard *et al.*[30] TGF-β2, activin, and inhibin were unreactive in the assay when tested at concentrations of 500–1000 ng/ml.

In order to develop such an assay, which depends on optimal concentrations of antibodies, there is first a need to titer the antibodies in a checkerboard fashion, as described.[28] Several concentrations of coat antibody are titered against several dilutions of the enzyme-conjugated antibody, using a standard curve range (typically 200–0.56 ng/ml). Maximal sensitivity is usually achieved by decreasing the amount of antibody used for coating while increasing the concentration of enzyme-conjugated antibody. Many double-antibody ELISAs are linear between 1 and 50 ng/ml of the peptide to be analyzed with antibody concentrations of 0.5 to 2 μg/ml used to coat the microtiter plates. Optimal capture antibody and conjugate dilutions are those which after a fixed reaction time (20–30 min) will allow maximal sensitivity and the highest absorbance within the linear range of the spectrophotometer (an absorbance of 2.0 is the upper limit of linearity of most instruments). ELISAs such as these are easily automated and provide high throughput analysis of large number of samples assayed in duplicate.

Conjugation of MAb 4A11 to horseradish peroxidase (HRP; Boehringer Mannheim, Indianapolis, IN) is performed essentially according to the method of Nakane and Pierce.[29] This method is based on the periodate oxidation of hydroxyl groups in the carbohydrates of the enzyme to aldehyde groups, followed by the formation of Schiff bonds between the newly formed aldehyde groups and the amino groups of the antibody. The cross-link is subsequently stabilized by reduction with $NaBH_4$. HRP is activated by adding 200 μl of freshly prepared 0.1 *M* $NaIO_4$ solution to 4 mg HRP in 1 ml water for 20 min at ambient temperature. The activated HRP is then chromatographed on Sephadex G-50 (Pharmacia), preequilibrated with 1 m*M* sodium acetate, pH 4.0, to avoid self-coupling of HRP (which would occur if the solution were kept at alkaline pH). The colored HRP peak collected from the chromatography column is adjusted to pH 9.5 with 0.2 *M* sodium carbonate just prior to adding it to the antibody. The antibody (MAb 4A11) at 2–5 mg/ml in coupling buffer (10 m*M* sodium carbonate, pH 9.5) is added to the activated HRP in a weight ratio of antibody to HRP of 1–2 : 1. The coupling reaction is performed at ambient temperature

[30] D. Rodbard, P. J. Munson, and A. DeLean, *in* "Radioimmunoassay and Related Procedures in Medicine," Vol. 1, p. 469. Proceedings of an International Symposium on Radioimmunoassay and Related Procedures in Medicine, International Atomic Energy Agency, Vienna, Austria, 1978.

for 2 hr, then stopped by adding 100 μl of freshly prepared 4 mg/ml $NaBH_4$ for 2 hr at 4°.

For the quantitation of TGF-β1, MAb 12H5 is added at 0.5 μg/ml (100 μl/well) in coating buffer (50 m*M* carbonate/bicarbonate, pH 9.6) to 96-well microtiter plates for an overnight incubation at 4°. The plates are then washed 3 times with wash buffer (PBS, 0.05% Tween 20) and blocked for 1 hr at ambient temperature with ELISA diluent (PBS, pH 7.4, 0.5% BSA, 0.05% Tween 20, and 0.01% thimerosal). They are then washed again 3 times with wash buffer, and 100 μl of each TGF-β1 standard (40 to 0.63 ng/ml), control, or sample in ELISA diluent is added to duplicate wells for a 2-hr incubation at ambient temperature. The plates are subsequently washed 3 times with wash buffer and incubated for 1 hr at ambient temperature with HRP-conjugated MAb 4A11, freshly diluted to its optimal concentration in ELISA diluent. After 3 subsequent washes with wash buffer, substrate solution (2.2 m*M* *o*-phenylenediamine substrate in 50 m*M* sodium phosphate, 0.1 *M* sodium citrate, pH 5.0, containing 0.01% H_2O_2) is added for 30 min at ambient temperature (100 μl/well), and the reaction is stopped with 100 μl/well of 4.5 *M* H_2SO_4. Optical densities at 492 nm are then read with an automatic plate reader (Molecular Devices, Menlo Park, CA). Since this is a noncompetitive assay, the optical density is directly proportional to the antigen concentration. Data are reduced using a four-parameter curve-fitting program based on an algorithm for least-squares estimation of nonlinear parameters[19] to provide antigen concentrations calculated from the standard curve.

The interassay precision was good, with percent coefficients of variation of 8–10% for TGF-β samples assayed in nine different plates. The sensitivity and precision of this assay compared well with values obtained with the radioimmunoassay and the radioreceptor assay, with the obvious advantage of not requiring the use of radioactive reagents. This assay was only possible with the availability of monoclonal antibodies to TGF-β1. Many combinations of polyclonal antibodies were previously tried with complete lack of success, in contrast to the recently reported ELISA based on polyclonal antibodies to TGF-β.[20] The assay is adaptable to the measurement of TGF-β in cell culture fluid or in serum or plasma, although these fluids may contain interfering substances or binding proteins. Treatments such as acidification, partial denaturation, and/or extraction may therefore be needed prior to quantitation of the TGF-β.

Bioassay for Transforming Growth Factor β

The multifunctionality of TGF-β has resulted in the development of many different types of cell-based assays to measure its activity.[2] Since

TGF-β can either inhibit or enhance cellular proliferation, depending on the cell type, assays can be developed which measure either property. To measure the growth-promoting or mitogenic effects of TGF-β, the assay system most generally used is the stimulation of proliferation of fibroblastic cell types such as AKR-2B (mouse embryo fibroblasts) or NRK cells (normal rat kidney fibroblasts) in soft agar. On the other hand, the growth-inhibitory effects of TGF-β can be measured on a variety of both normal and transformed cell types grown either as monolayers or in soft agar. These include hepatocytes, endothelial cells, lymphocytes, fibroblasts, and epithelial cells.

One of the most sensitive *in vitro* assays to detect TGF-β bioactivity is based on the antiproliferative effects of TGF-β on mink lung epithelial-like cells (Mv1Lu).[20] This cell line is available from the American Type Culture Collection (Rockville, MD; ATCC CCL64). The parental cell line was cloned by limiting dilution at 0.3 cells/well. Clone 3D9 (designated Mv3D9) was chosen from 78 individual clones because of its greater sensitivity to the growth-inhibitory effects of TGF-β1 when compared to the parental cell line. Mv3D9 cells are maintained *in vitro* by weekly passage in CMEM consisting of Eagle's minimum essential medium supplemented with 0.1 m*M* nonessential amino acids, 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO), and 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories, Logan, UT).

The TGF-β assay utilizing these cells is based on the ability of TGF-β to inhibit [^{3}H]thymidine uptake by Mv3D9 cells. The assay is performed as follows: samples to be assayed (100 μl/well) are serially diluted in assay medium (CMEM supplemented with 0.1% FBS) into 96-well flat-bottomed microtiter plates, followed by the addition of 10^4 Mv3D9 cells/well in 100 μl of assay medium. Prior to addition to the wells, assay samples which contain inactive forms of TGF-β are acid-activated by incubating each 100-μl sample with 10 μl of 1.2 *N* HCl for 15 min at ambient temperature and neutralized with 20 μl of 0.5 *M* HEPES buffer containing 0.72 *M* NaOH. The plates are then incubated at 37° with 5% CO_2 for 24 hr. For the last 4 hr of culture, 20 μl/well (1 μCi) of [^{3}H]thymidine in CMEM (6.7 Ci/mmol, Amersham) is added. After incorporation of the label, 100 μl of 0.5% trypsin/5.3 m*M* EDTA solution (GIBCO) is added to each well, and the plates are incubated for an additional 15 min at 37°. Cells are then harvested onto glass fiber filters and counted in a liquid scintillation counter. Results (pg/ml of TGF-β) are calculated based on percent inhibition of thymidine incorporation compared with a recombinant human TGF-β1 laboratory standard. A typical dose–response is in the range of 2–250 pg/ml TGF-β.

Monoclonal Antibody Neutralization of Transforming Growth Factor β *In Vitro*

To test whether antibodies (polyclonal or monoclonal) to TGF-β have neutralizing activity *in vitro*, TGF-β at a constant concentration (1000–2000 pg, final concentration) in CMEM is preincubated overnight at 4° with a dose titration of 0.39–100 μg/ml (final concentration) of the antibodies before being tested in the bioassay described above. Mv3D9 cells (10^4/100 μl/well, see above) are then added to each 100-μl sample for 20 hr at 37°. The cells are pulsed for 4 hr at 37° with 1 μCi/20 μl/well of radiolabeled thymidine, harvested as described above, and counted in a scintillation counter. The reversal of TGF-β inhibition of thymidine uptake by the test antibody is compared to that of a negative control MAb preincubated with TGF-β (we used anti-human growth hormone MAb 6G12 from Genentech Inc., South San Francisco, CA).

Conclusion

The development of antibodies and assays for transforming growth factor β has been a difficult endeavor. Although platelets are an abundant source of material for purified TGF-β,[1,31,32] antibodies of high titer and neutralizing antibodies were difficult to obtain, presumably owing to the small size and conserved amino acid sequence of this protein. Some authors successfully produced monoclonal antibodies to TGF-β with conventional immunization and fusion protocols[21]; however, we had to immunize mice in the footpads and fuse cells from their lymph nodes to obtain these reagents. In the absence of monoclonal antibodies, a conventional radioimmunoassay was developed with polyclonal antibodies to TGF-β, and we found that the radioiodination of TGF-β was a procedure which required careful handling of the molecule. Provided attention was paid to the particular behavior and properties of TGF-β, we were able to produce good reagents and reliable procedures for the measurement of this growth factor.

[31] C. B. Childs, J. A. Proper, R. F. Tucker, and H. L. Moses, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5312 (1982).
[32] R. K. Assoian and M. B. Sporn, *J. Cell Biol.* **102**, 1217 (1986).